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Stacking anabolic androgenic steroids (AAS) during puberty in rats: A neuroendocrine and behavioral assessment

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Abstract

Anabolic androgenic steroid (AAS) abuse is increasing in teenagers. We examined the effects of stacked AAS in adolescent male rats. Stacking, in which multiple AAS are taken simultaneously, is commonly employed by humans. Beginning at puberty gonadally intact male rats received testosterone, nandrolone, or stanozolol. Additional groups received stacked AAS: testosterone + stanozolol, nandrolone + stanozolol, or nandrolone + testosterone. Injections continued during tests for sexual behavior, vocalizations, scent marking, partner preference, aggression and fertility. Body and reproductive tissue weights were taken. Sexual and aggressive behaviors were increased by testosterone yet inhibited by stanozolol; nandrolone had no effect. Stacking testosterone with stanozolol prevented the inhibitory effects of stanozolol. Body weight was decreased by testosterone and all stacked AAS. Cell nuclear androgen receptor binding in brain was significantly increased in nandrolone males and decreased in stanozolol males; testosterone males were slightly higher than controls. Androgen receptors in stacked groups were intermediate between individual AAS suggesting that stanozolol competed with other AAS for androgen receptors despite its low affinity. The results indicate that stacking AAS influences the effects of individual AAS on behavioral and endocrine measures, and levels of androgen receptor occupation are not directly correlated with AAS effects on behavior.

Keywords: Aggression; Androgen receptor; Nandrolone; Puberty; Sexual behavior; Stacking; Stanozolol; Steroids; Testosterone

1. Introduction

Anabolic androgenic steroid (AAS) abuse is a prevalent social problem (Yesalis and Bahrke, 2000; Johnston et al., 2004). Despite the illegality and known adverse effects surrounding AAS abuse, over 1 million Americans have used AAS (Pope et al., 2000; Johnston et al., 2004). Of particular concern is the rising popularity of AAS with adolescents (Anderson et al., 1997; Bahrke et al., 1998; DuRant et al., 1995; Faigenbaun, 1998; Johnston et al., 2004; Yesalis and Bahrke, 2000). It is now known that puberty is a second critical period during which gonadal steroid hormones organize and activate neural circuits (Sisk et al., 2003). Thus, chronic AAS exposure during adolescence could alter the development of hormone sensitive neural systems ultimately affecting behavioral maturation (McGinnis, 2004).

AAS, including the endogenous male reproductive hormone, testosterone, as well as synthetic analogues of androgens, act by binding to androgen receptors (Krey and McGinnis, 1990; Roselli, 1998). In adult males, androgens modulate male sexual behavior (Meisel and Sachs, 1994) and aggression (Barfield et al., 1972), and maintain reproductive tissues (Bardin and Catterall, 1981). The behavioral effects of three commonly abused AAS, testosterone, nandrolone, and stanozolol have been studied (Clark and Henderson, 2004) in both adult and adolescent animals. Chronic exposure to high levels of testosterone increases male sexual and aggressive behaviors (Lumia et al., 1994; McGinnis et al., 2002a,b), nandrolone has been shown to increase (Long et al., 1996 Johansson et al., 2000) and to have no effect (Breuer et al., 2001; McGinnis et al., 2002a,b) on aggression, and stanozolol inhibits male sexual and aggressive behaviors (McGinnis, 2004). Studies on the effects of exposure to individual AAS have been important for assessing AAS mechanisms, and provide a foundation for understanding the effects of stacking AAS.

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Stacking, or the simultaneous administration of two or more AAS, is a common AAS administration strategy employed by humans (Strauss and Yesalis, 1991; Yesalis and Bahrke, 1995). Animal studies using an AAS 'cocktail' to mimic stacking (DeLeon et al., 2002; Grimes and Melloni, 2002; Grimes et al., 2003) have been valuable in demonstrating that AAS exposure increases aggression. However, these cocktails did not include the AAS stanozolol. Because of its high anabolic (muscle-building) and lower androgenic properties, stanozolol is frequently used in AAS stacking paradigms by humans (Mottram and George, 2000). However, stanozolol is not aromatized to an estrogen and has been shown to inhibit androgen-dependent behaviors in animal models (Martinez-Sanchis et al., 1996; Clark et al., 1997; Breuer et al., 2001; McGinnis et al., 2002a,b). Both testosterone and estrogen may be important for pubertal development (Sisk et al., 2003). As a result, suppressing endogenous testosterone, and its metabolism to estradiol, by exposure to stanozolol during puberty may have a negative impact on normal behavioral maturation. It is therefore important to understand the implications of stacking AAS in adolescents (Burnett and Kleiman, 1994; Johnson, 1990; Rogol and Yesalis, 1992).

One rationale for stacking is that a synergistic effect may be achieved by combining specific AAS (Rogol and Yesalis, 1992). Although the doses typically used in animal studies are very high, it is not known whether stacking will exaggerate the behavioral consequences of pubertal AAS exposure. In the current study we chose to give double the AAS dose in the stacked groups for several reasons. First, we wanted to be able to make meaningful correlations between the impact of AAS on behavior and the effect of AAS on androgen receptor occupation. This would not be possible if the doses were different. Second, the double dose would more closely approximate how humans stack AAS. Human AAS users self-administer AAS 10–100 times serum testosterone levels (Mottram and George, 2000) without regard to maintaining a constant dose. Finally, the 10 mg/kg dose would show whether doubling the AAS dose would potentiate the behavioral effects of individual AAS.

To address these problems, this study had two primary goals. The first goal was to systematically assess the effects of stacking testosterone, nandrolone, and stanozolol on male sexual, sociosexual (partner preference, 50-kHz vocalizations, and scent marking) and aggressive behaviors, as well as male reproductive tissues. The second goal was to correlate the behavioral effects of AAS with androgen receptor occupation in the brain.

2. Materials and methods

2.1. Subjects

Male Long–Evans rats used as experimental subjects were obtained at 35 days of age. Adult female Long–Evans rats (225–250 g) were used as stimulus and fertility females. Additional male Long–Evans rats of similar weights to the experimental males were used as aggression test opponents or androgen receptor assay animals. All animals were purchased from Charles River Laboratories (Wilmington, MA). Experimental and opponent males were housed individually in 43 × 26 × 20 cm clear Nalgene cages, with stimulus females housed in groups of three. A reverse 12:12, light:dark schedule was maintained, with lights going off at noon. The temperature and humidity of the animal and behavior testing rooms were held at approximately 23 °C and 73% respectively, with food and water available ad libitum. Animals were cared for in accordance to the guidelines established by the National Institutes of Health.

2.2. AAS exposure

All experimental males were gonadally intact. Experimental animals were given an AAS regimen beginning at the onset of puberty (postnatal day 41, the day after preputial separation) (Korenbrot et al., 1977; Feinberg et al., 1997) and continued to receive injections (5 days/wk) until sacrifice. Six AAS treatment groups and one vehicle control group (polyethylene glycol-200) were used. Three treatment groups were injected with individual AAS (5 mg/kg): either testosterone (4-androsten-17β-ol-3-one 17-propionate), nandrolone (17β-hydroxy 19-norandrost-4-en-3-one), or stanozolol (17β-hydroxy-17α-methyl-androstano [3,2-c]pyrazole) for comparison with stacked groups. The three additional groups received stacked AAS: testosterone + stanozolol, nandrolone + stanozolol, or nandrolone + testosterone (5 mg/kg each) for a total dose of 10 mg/kg. All AAS were purchased from the Sigma-Aldrich Co. (St. Louis, MO).

2.3. Behavioral tests

All tests were performed within the first 6 h of the dark period, with a dim red light illuminating the testing room for observational purposes. At postnatal day 57, all experimental animals received a 30-min exposure to a receptive female to provide an opportunity for sexual experience. The order of testing and ages of experimental animals at time of testing are shown in Fig. 1. In pilot studies we determined that male rats exhibit more vocalizations and scent marks, and a greater preference for a receptive female if they have been previously exposed to a sexually receptive female. So the test for sexual behavior was given first. Aggression tests began on day 67. The animals were tested for aggression with and without physical provocation (tail pinch). The physical provocation test was last to prevent any interactive effects that might occur with regard to the other behaviors.

2.4. Sexual behavior tests

At postnatal day 70, treatment males were placed in individual 10-gallon glass aquariums with a sexually receptive female for a test of sexual behavior. Ovariectomized females implanted with 5 mm long estradiol-filled silastic capsules were brought into behavioral estrous by an injection of 500 μg progesterone 4 h prior to testing. Stimulus females were rotated every 5 min to randomize the females' influence on the test (Harding and McGinnis, 2003; Vagell and McGinnis, 1998).

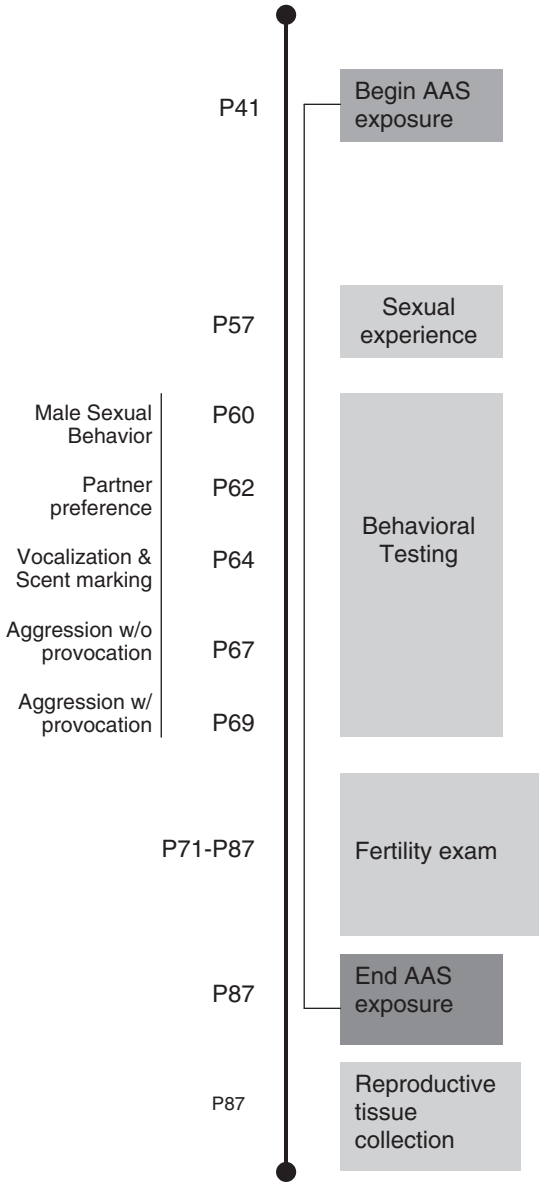

Fig. 1. Experimental timeline depicting the schedule of AAS injections, behavioral testing, fertility testing, and reproductive tissue weight collection. P = postnatal day.

Behaviors scored included mounts, mount latency, intromissions, intromission latency, ejaculation, ejaculation latency, and postejaculatory refractory interval. If a treatment male failed to exhibit sexual behavior within the first 15 min as defined by the display of a mount or intromission, the test was terminated. Males were given 30 min after the display of a mount or intromission to achieve an ejaculation. If the male ejaculated he was given 15 min to display a mount or intromission. This completed an ejaculatory series.

2.5. Partner preference tests

Partner preference tests were conducted in a three-chambered apparatus (91 cm wide × 62 cm deep × 40 cm tall), with each chamber being of equivalent dimensions (29 cm wide × 62 cm deep). One outer chamber contained a sexually receptive female, the center chamber remained empty, while the other outer chamber housed a non-receptive ovariectomized female. The experimental male was placed into the empty center chamber and had equal access to all three chambers. The sexually receptive and ovariectomized females were secured in their chambers by cylindrical wire mesh enclosures (8 in diameter × 5 in high) which prohibited sexual contact, but allowed olfactory, visual, and auditory stimulation.

The time spent in each chamber over a 10-min test was measured by an observer using a computer program for place preference testing (Vagell et al., 1995). The presence of a male in a chamber was defined by the contact of all four paws within the specific chamber. The time spent with the ovariectomized female was subtracted by the time spent with the sexually receptive female (Vagell and McGinnis, 1997; Harding and McGinnis, 2003). A positive score indicated a preference for the sexually receptive female.

2.6. Scent marking and vocalization tests

The scent marking and 50-kHz ultrasonic vocalization test began with a sexually receptive female confined to one side of a removable wire grid, with the treatment male and the scent marking jar on the other. After a 3-min sensory acquisition phase, the receptive female along with the grid divider was removed from the testing arena (McGinnis and Vakulenko, 2003). Both scent markings and vocalizations were then recorded for 5 min. A scent mark was scored when the male climbed over the jar, rubbing his perineum along the surface, leaving visible traces of an aqueous scent mark. The jar was cleaned thoroughly with warm water and dried prior to the testing of each male. Ultrasonic vocalizations were recorded by an observer with headphones using an ultrasonic frequency bat detector set to 50-kHz (model U30, Ultrasound Advice, London, U.K.), connected to a capacitance microphone (Vagell and McGinnis, 1998).

2.7. Aggression tests

For aggression testing experimental males were housed in their cages and omitted from routine cage cleaning for at least one week prior to testing to establish home cage familiarity (Barfield et al., 1972). During the tests the metal cage tops were replaced with an inverted clear plastic cage with the bottom removed. This heightened arena allowed ample room for the rats to rear and move freely, yet preventing escape. All aggression tests were 10 min in duration, with experimental males in their home cages. Males received two tests for aggression 48 h apart. The first test was without physical provocation. The second test was with physical provocation using a mild tail pinch to the distal end of the experimental male's tail, 1 pinch/min throughout the test (Smith et al., 1997; McGinnis et al., 2002a,b; Farrell and McGinnis, 2004). No treatment male was paired against the same opponent more than once, nor did any opponent male fight in two consecutive tests. Behaviors were recorded only when initiated by the experimental animal

and included mounts, dominance postures, threats, and attack/fight sequences, in concordance with our standard operational definitions (Breuer et al., 2001; Farrell and McGinnis, 2003a,b, 2004). A composite aggression score was obtained by adding all of the aggressive behaviors displayed by the experimental male (Christie and Barfield, 1979; Farrell and McGinnis, 2004).

2.8. Fertility tests

To assess fertility, experimental males (P80) were placed in a cage with a gonadally intact female for the duration of three estrous cycles (Apx. 12 days). The male was then removed and the female was housed through one gestation cycle (Apx. 22 days). Whether or not the female birthed pups was recorded. Following completion of the fertility test, experimental subjects were euthanized with an overdose of chloral hydrate and the testes, ventral prostates, and seminal vesicles were excised and weighed for analysis.

2.9. Cell nuclear androgen receptor binding assays

Androgen receptor assays were used to assess the level of androgen receptor occupation in rats exposed to stacked AAS. Because of technical problems, it was necessary to conduct the assays on separate groups of rats. The animals were the same age and body weight range as the experimental males at the time of sacrifice. Ten rats in each group were treated with single AAS (5 mg/kg), stacked AAS (5 mg/kg+5 mg/kg), or vehicle. AAS injections were given for 8 days prior to sacrifice to suppress endogenous testosterone and the animals were sacrificed 1–2 h after the last AAS injection to ensure that measures of maximal androgen receptor occupation would be obtained (Krey and McGinnis, 1990). The methods for measurement of cell-nuclear androgen receptors have been previously described (Krey and McGinnis, 1990; McGinnis et al., 1983; McGinnis and Mirth, 1986; Vagell and McGinnis, 1997, 1998). All animals received an overdose of chloral hydrate prior to decapitation as pilot studies indicated that this anesthesia does not affect androgen receptor binding. The brains were rapidly removed and placed on ice. Tissue was dissected and combined from the hypothalamus, preoptic area, amygdala, and septum (McGinnis et al., 1983). Brain samples from two rats were pooled to provide sufficient tissue for analysis. Thus, brains from ten rats yielded five determinations ($n=5$).

Tissues were homogenized and cell nuclei purified by sucrose gradient density centrifugation. Receptor complexes in the nuclear pellet were salt extracted and incubated overnight with 4 nM [^{3}H]R1881 (methyltrienolone: specific activity 87 Ci/mmol; PerkinElmer Inc., Boston, MA) with and without 100-fold molar excess dihydrotestosterone to correct for nonspecific binding. Bound steroids were separated from free steroids by gel filtration using Sephadex LH-20 chromatography (Pharmacia Biotech, Uppsala, Sweden). Bound radiolabeled steroid was measured using scintillation counting. DNA content in the nuclear pellet was determined by the method of Burton (1956), and values expressed as fmoles/mg of DNA.

2.10. Statistical analysis

All data recorded were entered into StatView v5.0 (Abacus Concepts Inc., Berkeley, California) for analysis. Initial analyses for all behavioral data (except for the percent of males ejaculating, mounting, and intromitting), reproductive tissue weights data, and AR assay data, began with ANOVA to determine overall effects, followed by Fisher's PLSD for post hoc analyses. Percentage data obtained during the test for male sexual behavior was analyzed using chi-square tests to assess overall effects. Planned post hoc comparisons were made using Fisher's exact probability test.

3. Results

The effects of AAS on the male sexual behavior of ejaculation are shown in Fig. 2. Chi-square analysis on the percentage of males ejaculating during the test of male sexual behavior yielded an overall effect ($\chi^2(6, N=71)=29.223$, $p<.0001$). Due to this overall difference between groups, Fisher's exact analyses were performed on individual AAS groups in comparison to control animals, and on stacked AAS groups in comparison to animals receiving the individual AAS contained in their stacked treatment. Significantly fewer stanozolol males ejaculated compared to controls ($p=.012$) whereas significantly more testosterone males ejaculated compared to controls ($p=.034$). No significant effect of nandrolone in comparison to controls was found. Analysis of the testosterone + stanozolol group in comparison to testosterone alone indicated that the effects of testosterone on ejaculation were not decreased when testosterone was stacked with stanozolol, with testosterone greatly

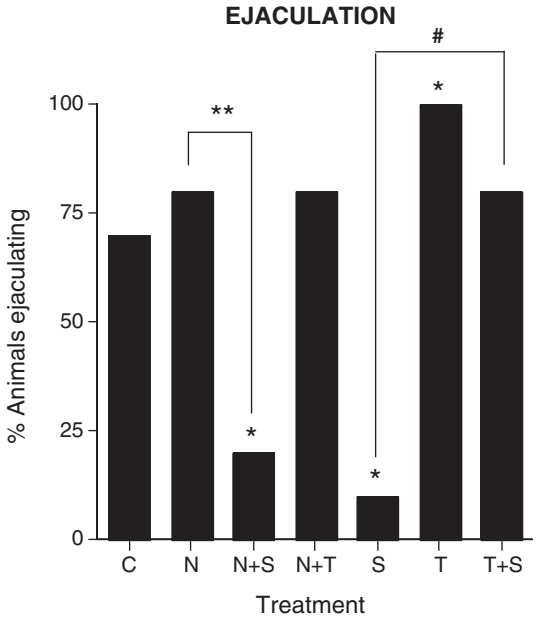

Fig. 2. Percent of males ejaculating in each treatment group. C = control, N = nandrolone, N + S = nandrolone + stanozolol, N + T = nandrolone + testosterone, S = stanozolol, T = testosterone, T + S = testosterone + stanozolol. Fisher's exact probability test *$p<.05$ in comparison to controls, **$p<.01$, $^{\#}p=.0017$ as indicated.

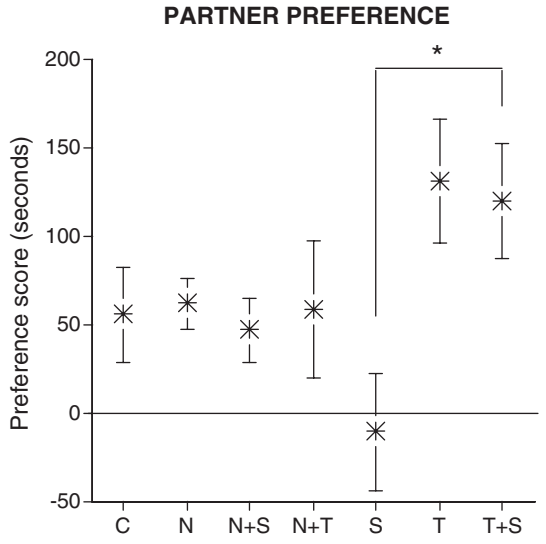

Fig. 3. Mean partner preference score±SEM for each treatment group. Preference was determined by subtracting the time spent with an ovariectomized female from time spent with a receptive female. C = control, N = nandrolone, N + S = nandrolone + stanozolol, N + T = nandrolone + testosterone, S = stanozolol, T = testosterone, T + S = testosterone + stanozolol. Fisher's PLSD analysis *$p<.05$.

overriding the inhibitory effects of stanozolol on male sexual behavior ($p=.002$). In contrast, nandrolone + stanozolol males ejaculated significantly less than controls ($p=.044$) and males treated with nandrolone alone ($p=.007$). ANOVA and post hoc analyses of frequencies and latencies of sexual behaviors from those animal that behaved, showed that there were no significant differences between groups (data not shown).

ANOVA analysis of the total amount of time spent with the sexually receptive female versus the ovariectomized female (Fig. 3) resulted in a significant overall effect ($F(6, 64)=2.519$, $p=.030$). However, Fisher's PLSD post hoc test showed that no AAS group significantly differed from control. Since the goal of this study is to determine the role of individual AAS in a stacked paradigm, one obvious point of interest was the large difference in preference scores between the testosterone + stanozolol and stanozolol groups. Fisher's PLSD analysis comparing these groups showed that stacking testosterone + stanozolol significantly prevented the negative preference produced by stanozolol alone ($p=.012$).

Results for the scent marking tests are presented in Fig. 4. ANOVA analysis revealed a significant group effect on scent marking ($F(6, 59)=3.895$, $p=.002$). Fisher's PLSD showed that animals exposed to either nandrolone or testosterone were similar to controls ($p>.5$). However, stanozolol males scent marked significantly less than controls ($p=.001$). The stanozolol-induced decrease was prevented when stanozolol was combined with testosterone, resulting in a significant ($p<.0001$) increase in scent marks in the testosterone + stanozolol group compared to the stanozolol group. The effects of nandrolone on scent marking were significantly decreased ($p=.013$) when nandrolone was stacked with stanozolol, and when nandrolone was stacked with testosterone ($p=.049$).

Results for the 50-kHz ultrasonic vocalization tests are shown in Fig. 4. Vocalizations were significantly decreased in stanozolol males ($p<.0001$), and as with scent marks, this effect was prevented by combining stanozolol with testosterone ($p=.002$). Combining nandrolone with stanozolol also prevented the inhibitory effects of stanozolol on vocalizations ($p=0.5$). Interestingly, nandrolone + testosterone treatment resulted in a

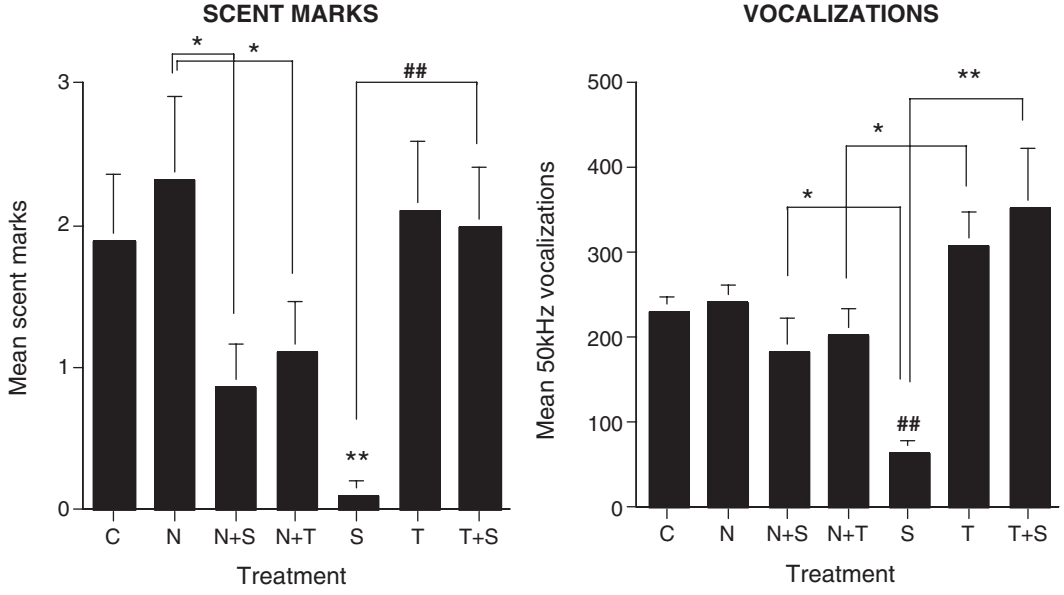

Fig. 4. Results from scent marking and 50-kHz ultrasonic vocalization tests. C = control, N = nandrolone, N + S = nandrolone + stanozolol, N + T = nandrolone + testosterone, S = stanozolol, T = testosterone, T + S = testosterone + stanozolol. C = control, N = nandrolone, N + S = nandrolone + stanozolol, N + T = nandrolone + testosterone, S = stanozolol, T = testosterone, T + S = testosterone + stanozolol. Values are expressed as means±SEM. Fisher's PLSD analysis *$p<.05$, **$p<.01$, ##$p<.0001$, compared to controls unless otherwise indicated by treatment comparison lines.

significant decrease ($p=.021$) in vocalizations compared to testosterone alone.

Aggression was higher than controls in testosterone males, but was only significantly increased following tail pinch ($p<.0001$) (Fig. 5). Similar to testosterone males, testosterone + stanozolol males were more aggressive than controls only when tail-pinched ($p=.005$). Males receiving stanozolol alone displayed decreased levels of aggression compared to controls ($p=.009$) following tail pinch.

Fig. 6 shows the reproductive tissue weights for all treatment groups. Fisher's PLSD analysis showed that the testes from all AAS groups except nandrolone males ($p=.053$) weighed significantly less than controls ($p\leq.001$). Seminal vesicle weights were significantly increased by exposure to nandrolone + testosterone ($p<.0001$), testosterone + stanozolol ($p<.01$) and to testosterone alone ($p<.05$). However, seminal vesicle weights were significantly decreased from those of controls in nandrolone ($p<.01$), nandrolone + stanozolol ($p<.01$) and stanozolol males ($p<.0001$). Ventral prostate weights were significantly increased by chronic pubertal exposure to nandrolone + testosterone ($p<.0001$) and testosterone + stanozolol ($p<.001$).

No significant group effects of AAS on fertility were found through Fisher's PLSD analysis. In particular, the number of males able to sire pups in each group was: control=11/11, testosterone=10/10, nandrolone=7/10, stanozolol=9/10, testosterone + stanozolol=7/10, nandrolone + stanozolol=9/10, and nandrolone + testosterone=10/10.

Fisher's PLSD were employed to assess specific effects of AAS exposure on weekly body weights (Table 1). These post hoc tests showed that pubertal males chronically exposed to testosterone and testosterone + stanozolol all showed significant ($p<.05$) decreases in body weight starting on the fourth week of

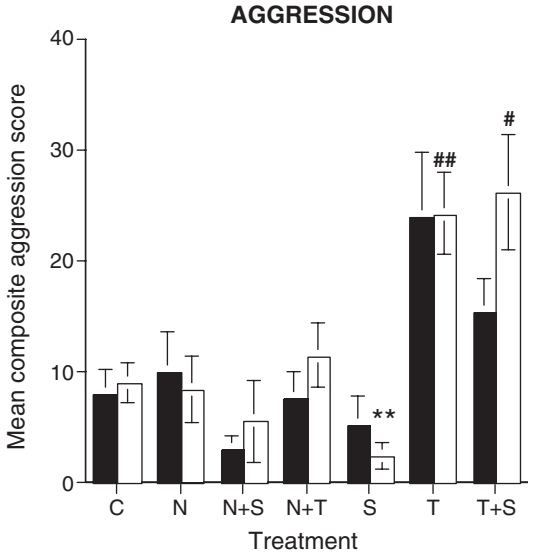

Fig. 5. Mean (± SEM) composite aggression scores from the non-tail pinch test (solid bars) and tail pinch test (open bars). C = control, N = nandrolone, N + S = nandrolone + stanozolol, N + T = nandrolone + testosterone, S = stanozolol, T = testosterone, T + S = testosterone + stanozolol. Fisher's PLSD analysis $^{**}p<.01$, $^{\#}p<.001$, $^{\#\#}p<.0001$ in comparison to respective controls.

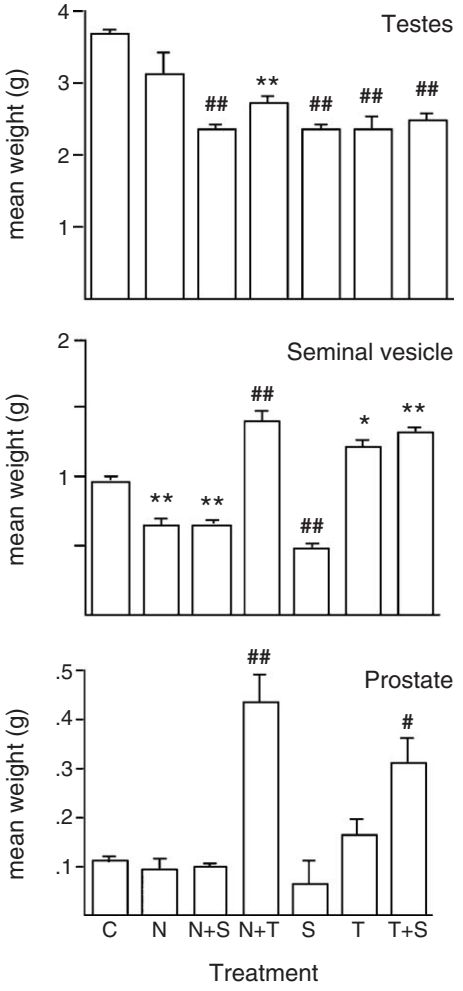

Fig. 6. Mean (± SEM) tissue weights for testes, seminal vesicles, and ventral prostates from gonadally intact experimental male rats, taken at postnatal day 87. C = control, N = nandrolone, N + S = nandrolone + stanozolol, N + T = nandrolone + testosterone, S = stanozolol, T = testosterone, T + S = testosterone + stanozolol. Number of animals per group for controls = 11 and for all others = 10. $^{*}p<.05$; $^{**}<.01$; $^{\#}p<.001$; $^{\#\#}p<.0001$. All statistical comparisons were made to gonadally intact controls.

treatment. The body weights of these animals remained low for the remainder of the study and became increasingly different from controls over time. A similar decrease in body weights of nandrolone + testosterone males was found beginning at week 5, and in the nandrolone + stanozolol group beginning at week 8.

The effects of AAS treatment on androgen receptor occupation in the brain (Fig. 7) were analyzed with ANOVA, followed by Fisher's PLSD for significant effects. Initial analyses yielded a significant overall effect on androgen receptor occupation ($F(6, 28)=6.025$, $p=.001$). Post hoc tests revealed significant increases in androgen receptor occupation in nandrolone ($p=.028$) and nandrolone + testosterone ($p=.021$) AAS groups compared to gonadally intact controls. In contrast, stanozolol exposure resulted in a significant decrease in androgen receptor occupation compared to controls ($p=.046$). No cumulative effects of AAS were seen in any stacked group (e.g., nandrolone + testosterone did not produce effects on androgen receptor occupation equal to the effects of testosterone added to the

Table 1
Weekly body weights of AAS males

Treatment	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
C (n=11)	347±7	376±9	404±13	428±11	454±10
N (n=10)	335±8	363±7	393±10	417±13	440±13
N + S (n=10)	325±11	358±11	385±12	404±15	422±15*
N + T (n=10)	326±6	349±6*	369±4*	385±6**	391±7$^{\#}$
S (n=10)	345±6	377±6	403±9	420±11	443±11
T (n=10)	322±6*	342±5**	360±7**	382±7**	405±7**
T + S (n=10)	319±8*	339±6**	357±7**	369±8$^{\#}$	379±6$^{\#\#}$

Body weights are expressed in grams, as means±SEM.
Wk 4=fourth week of AAS exposure.
C = control, N = nandrolone, N + S = nandrolone + stanozolol, N + T = nandrolone + testosterone, S = stanozolol, T = testosterone, T + S = testosterone + stanozolol.
Body weights from weeks 1–3 were not significant in comparison to controls (data not shown).
Fisher's PLSD analysis *p<.05, **p<.01, $^{\#}p$<.001, $^{\#\#}p$<.0001, versus control.

effects of nandrolone). In fact, in every stacked AAS group, the combined treatment resulted in androgen receptor values approximately midway between the two individual AAS.

4. Discussion

This is the first study to provide a comprehensive assessment of the effects of systemically stacking AAS. We focussed on pubertal animals rather than adults because altering the hormone environment during this developmentally sensitive period may have a more negative impact on behavior. Several androgen-dependent behaviors were measured, including sexual behavior, partner preference, ultrasonic vocalizations, scent marking, and aggression. We also obtained weekly body weights, weights for testes, prostate, and seminal vesicles, and determined fertility by cohabitation with a female. Finally, an exchange assay was used to assess androgen receptor occupation in brain cell nuclei. The AAS, testosterone, nandrolone, and stanozolol were selected because they are all highly abused (Mottram and George, 2000), have differing behavioral effects (McGinnis, 2004), and have differing affinities for the androgen receptor (Roselli, 1998).

Stacking testosterone with stanozolol was expected to prevent the inhibitory effects of stanozolol on androgen-dependent behaviors because stanozolol has a much lower affinity for the androgen receptor than testosterone. We found that testosterone prevented the inhibitory effects of stanozolol on every behavior measured. For example, only 10% of stanozolol males ejaculated, whereas 80% of testosterone + stanozolol males ejaculated. Likewise, scent marking and vocalizations of testosterone + stanozolol males were similar to testosterone-treated males and gonadally intact controls. Stanozolol-treated males failed to show a preference for a receptive female, but when stanozolol was combined with testosterone, partner preference was comparable to testosterone-treated males. Finally, males receiving testosterone + stanozolol showed a significant increase in aggression after provocation (mild tail pinch). This was similar to testosterone-treated males, and in marked contrast to stanozolol-treated males, which showed low levels of aggression. The hypothesis that stacking testosterone + stanozolol would prevent the inhibitory effects of stanozolol was borne out. However, the reason for this was apparently not due to the low affinity of stanozolol for the androgen receptor. It was predicted that because of stanozolol's low androgen receptor affinity, androgen receptor occupation in the stacked testosterone + stanozolol group would be similar to androgen receptor occupation in the testosterone alone group and would thus correlate with the behavioral findings. In fact, the level of androgen receptor occupation in the testosterone + stanozolol group was closer to stanozolol alone. Although androgen receptor action is no doubt necessary for AAS effects, and stanozolol clearly affects androgen receptor occupation, the lack of correlation between stanozolol's effects on behavior and androgen receptor's suggests that other, perhaps extranuclear interactions between testosterone and stanozolol occur, especially at high doses.

In contrast to the effects of testosterone, nandrolone, when stacked with stanozolol (nandrolone + stanozolol) prevented some, but not all of the inhibitory effects of stanozolol. Although partner preference and vocalizations in nandrolone + stanozolol males were similar to nandrolone-treated rats, significantly fewer nandrolone + stanozolol males ejaculated and scent marked compared to nandrolone males. There were no effects of nandrolone + stanozolol on aggression. This was not surprising since nandrolone alone and stanozolol alone did not enhance aggression, a finding that is consistent with our previous results (Farrell and McGinnis, 2003a,b, 2004). Our hypothesis that the inhibitory effects of stanozolol would be prevented when stacked with nandrolone was not confirmed as stanozolol clearly influenced many of the behaviors measured. Interestingly, androgen receptor occupation was significantly increased by nandrolone alone, and significantly decreased by stanozolol alone. However, when stacked, androgen receptor occupation was midway between the two. This indicates that, in

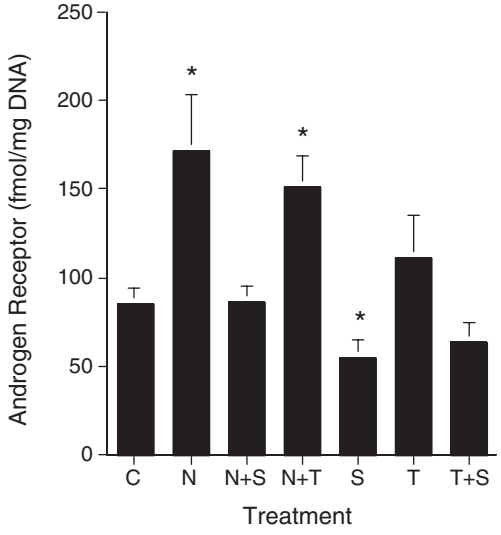

Fig. 7. Androgen receptor binding in combined hypothalamus, preoptic area, amygdala, and septum. Results are expressed as mean (± SEM) fmoles androgen receptor/mg DNA, n=5/group. C = control, N = nandrolone, S = stanozolol, T = testosterone.

spite of its low androgen receptor affinity, stanozolol competes with nandrolone for androgen receptor binding sites in neural tissue. Androgen receptor occupation was similar to gonadally intact control levels in both nandrolone + stanozolol and nandrolone + stanozolol males, but the two groups showed very different behavior patterns. This lack of correlation suggests that exposure to very high levels of AAS may have effects in neural tissue that do not normally occur in gonadally intact males with physiological levels of testosterone.

Since nandrolone and testosterone both have a high affinity for the androgen receptor, we postulated that stacking nandrolone + testosterone would enhance the effects of either AAS alone. This was not the case. In fact, with regard to behavior, the effects of nandrolone + testosterone were similar to nandrolone, not testosterone. Ejaculation and aggression were significantly increased by testosterone alone, but not by nandrolone alone, which has been reported previously (Farrell and McGinnis, 2003a,b, 2004). Combining nandrolone + testosterone resulted in levels of sexual behavior, partner preference, and vocalizations that were similar to nandrolone-treated males and to gonadally intact controls, though scent marks, which were similar in both nandrolone alone and testosterone alone males were significantly decreased in nandrolone + testosterone males. In any case, it is clear that the combined behavioral effect of nandrolone + testosterone is not additive, even though the AAS dose was double. It is also notable that a 10 mg/kg dose of AAS did not increase androgen receptor occupation beyond levels found with 5 mg/kg suggesting that all the receptors were occupied even at the 5 mg/kg dose. However, in the absence of an extensive saturation analysis, such a conclusion would be premature. In sum, there appears to be no direct correlation between the behavioral effects of stacking nandrolone + testosterone and androgen receptor binding.

The primary reason for including the groups receiving individual AAS was for comparison with stacked groups, since the behavioral effects of adolescent testosterone, nandrolone and stanozolol exposure have been previously demonstrated (Farrell and McGinnis, 2003a,b, 2004; Feinberg et al., 1997). One notable finding in the present study, was that significantly more (100%) of testosterone-treated males ejaculated, than controls (63%). An earlier study from this laboratory also reported that significantly more testosterone-treated males ejaculated compared to controls, also because fewer controls ejaculated (Feinberg et al., 1997). Two other studies found no differences in ejaculation (Farrell and McGinnis, 2003a,b, 2004), but virtually all the AAS and control males ejaculated. Since one cannot have more than 100% of males ejaculating, this ceiling effect may have masked a real difference between AAS and controls with regard to sexual behavior. The most likely explanation for these differences is the amount of prior sexual experience the animals had, a factor known to facilitate subsequent sexual behavior (Dewsbury, 1969). The two studies that found no differences in sexual behavior gave prior sexual behavior tests (Farrell and McGinnis, 2003a,b, 2004). The study finding that more testosterone-treated males ejaculated did not give the animals prior sexual experience (Feinberg et al., 1997). The rats in the current study received a 30-min exposure to females, which may not have been sufficient to induce copulation in the controls (Lopez et al., 1999). Further studies will be needed to confirm the interesting possibility that adolescent AAS exposure potentiates sexual behavior.

Reproductive tissue weights were taken as indices of the endocrine effects of AAS (Jarow and Lipshultz, 1990). With regard to testosterone + stanozolol, the pattern was the same as that found for behavior: stacking testosterone + stanozolol prevented all the inhibitory effects of stanozolol. For example, stanozolol-treated rats had seminal vesicle weights significantly below controls. Rats receiving testosterone + stanozolol had seminal vesicles and prostate weights that, like testosterone-treated males, were significantly higher than controls. Nandrolone, stanozolol, and nandrolone + stanozolol all significantly decreased seminal vesicle weight. Both stanozolol and nandrolone + stanozolol significantly decreased testes weight whereas nandrolone did not. However, nandrolone has previously been shown to suppress testes weight (Clark et al., 1997) so this may be a spurious effect. The results suggest that stacking nandrolone with stanozolol is essentially no different than either AAS alone. In contrast to the behavioral effects of nandrolone + testosterone, reproductive tissue weights were similar to testosterone-treated males rather than nandrolone-treated males. Seminal vesicle weights were highest in males exposed to testosterone whether alone or stacked with nandrolone or stanozolol. A striking increase in prostate weights of nandrolone + testosterone and testosterone + stanozolol groups allude to the possibility that AAS induces prostatic abnormalities, but such a conclusion is premature in the absence of histological evidence. The data show that nandrolone prevents the enhancing effects of testosterone with regard to behavior but does not prevent the effects of testosterone on reproductive tissue weight. While the reasons for this difference are not apparent, stacking these two AAS, nandrolone + testosterone does not have a greater effect than either AAS alone.

Despite the suppressive effects on reproductive tissue weights, and the low levels of male sexual behavior displayed by the nandrolone + stanozolol and stanozolol groups, no significant effects of AAS on fertility were found. This contrasts with results from a previous study (Farrell and McGinnis, 2003b), showing that stanozolol significantly decreased fertility in adolescent stanozolol-treated males receiving the same dose as the current study. One difference is that we allowed AAS males to cohabit with intact females for three estrous cycles, whereas Farrell and McGinnis (2003b) allowed cohabitation for two cycles. It is possible that this additional exposure to the female may have allowed for additional priming of neurotransmitter systems (Hull et al., 2004), eventually overriding the inhibitory effects of stanozolol.

Pubertal exposure to testosterone significantly reduced body weight as previously reported (Farrell and McGinnis, 2003b). We also found a significant decrease in the body weights of all stacked groups. Interestingly, this was the only cumulative effect of pubertal AAS exposure. Since the rats in the combined AAS groups received twice the dose (10 mg/kg/day total) compared to animals receiving individual AAS, we conclude

that the decreased body weight in the combined groups is due to the higher dose of AAS. This idea is supported by Lindblom et al. (2003) who reported that 15 mg/kg nandrolone suppressed body weight, whereas a 5 mg/kg dose did not. These authors also reported a dose-dependent suppression of proopiomelanocortin, and suggest that this may be related to the effects of AAS on body weight. The fact that the effect of AAS dose was found with body weight and not behavior, suggests that a higher dose of individual AAS would not be more efficacious than the 5 mg/kg used by most researchers (Clark and Henderson, 2004). However, this interesting question has not been directly tested.

Testosterone, nandrolone, and stanozolol all bind to neural androgen receptors, but with differing affinities (Roselli, 1998; McGinnis et al., 2002a,b). Moreover, the behavioral effects of testosterone are inhibited by androgen receptor blockers (Vagell and McGinnis, 1998). We hypothesized that androgen receptor occupation would be correlated with behavior, and that the effects of the lower affinity AAS, stanozolol, on androgen receptor occupation would be negligible when stacked with nandrolone or testosterone. Neither of these hypotheses were confirmed. As previously reported (McGinnis et al., 2002a,b; Roselli, 1998), nandrolone and testosterone exposure resulted in the highest levels of androgen receptor occupation and stanozolol exposure resulted in the lowest level of androgen receptor occupation. In spite of the high affinity for the androgen receptor, and the significantly increased androgen receptor occupation levels, the behavior of nandrolone-treated males was no different from gonadally intact controls. However, the inhibitory effect of stanozolol on androgen-dependent behaviors was consistent with the significantly lower level of androgen receptor occupation by stanozolol. Surprisingly, stanozolol reduced androgen receptor binding when stacked with nandrolone or testosterone. The ability of stanozolol to compete with nandrolone and testosterone for androgen receptors was unexpected in view of the low affinity of stanozolol for the androgen receptor. Moreover, this result is not consistent with the effectiveness of testosterone in preventing virtually all the inhibitory effects of stanozolol on behavior. Similar to testosterone + stanozolol, stanozolol + nandrolone and nandrolone + testosterone yielded androgen receptor binding levels which were midway between the levels of each AAS alone, and did not correlate directly with the behavioral effects of stacked AAS. In addition to stanozolol's decreased affinity for the androgen receptor, it is not aromatized to an estrogen (Winters, 1990). Since estrogen is involved in the expression of several androgen-dependent behaviors (Vagell and McGinnis, 1997, 1998; Bakker et al., 2004), at least some of the inhibitory effects of stanozolol may result from lack of estrogen, and not simply lower androgen receptor affinity.

The foregoing results suggest that the behavioral effects of AAS in pubertal rats cannot be explained by changes in classical androgen receptors. Other mechanisms, acting either separately or in concert with androgen receptors, may be involved. For example, stanozolol's inhibitory effects may be mediated by an extranuclear mechanism (Luzardo et al., 2000). Also, AAS exposure has been shown to allosterically modulate the function of the $GABA_A$ receptor (Bitran et al., 1993), a consequence that varies between different AAS (Clark and Henderson, 2004). It is important to keep in mind that reproductive function in gonadally intact male rats is maintained with only 40–60% of the androgen receptors occupied (Krey and McGinnis, 1990). The effects of prolonged receptor saturation are not known. In addition, high levels of AAS may result in binding to other steroid receptors such as glucocorticoid or estrogen receptors (Rockhold, 1993). Thus, it is possible that chronic exposure to very high doses of AAS may influence steroid receptor function in ways that do not exist in gonadally intact males.

Several significant findings emerged from this study. First, stacking testosterone, but not nandrolone, with stanozolol prevents the inhibition of male sexual, sociosexual, and aggressive behaviors by stanozolol. Previous animal studies have shown that pubertal exposure to stanozolol can have long lasting inhibitory effects on reproductive behaviors and tissues (McGinnis, 2004). Thus, stacking paradigms that do not include testosterone may impair behavioral maturation. Second, doubling the AAS dose did not result in a cumulative effect, suggesting that there are limits to the benefits to be derived from excessively high AAS doses. Third, our results illustrate that the behavioral and physiological consequences of AAS exposure are not directly correlated with classical AR action. Further studies on the mechanisms underlying AAS's powerful influences on behavior will aid in understanding the long-term impact of adolescent AAS use.

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